Current Biology 20, 49–54, January 12, 2010 ©2010 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.10.072

Report

Identification of MicroRNA Processing Determinants by Random Mutagenesis of *Arabidopsis MIR172a* Precursor

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Summary

MicroRNAs (miRNAs) are widespread posttranscriptional regulators of gene expression. They are processed from longer primary transcripts that contain foldback structures (reviewed in [1, 2]). In animals, a complex formed by Drosha and DGCR8/Pasha recognizes the transition between the single-stranded RNA sequences and the stem loop to produce the first cleavage step in miRNA biogenesis [3]. Whereas animal precursors are of uniform size and shape, their plant counterparts comprise a collection of variable stem loops, and little is known about the structural clues recognized during their processing. Here, we designed an unbiased approach based on the random mutagenesis of the *MIR172a* precursor to study miRNA processing in plants. Randomly mutated precursors were overexpressed in *Arabidopsis*, and their activity was determined in vivo. We gathered sequence data from these transgenes and used it to build a *MIR172a* precursor map highlighting relevant and neutral positions for its processing. A 15 nucleotide stem segment below the miRNA/miRNA* duplex was essential for *MIR172a* processing. In contrast, mutations in the terminal-loop region were mostly neutral, yet a loop was required for miR172 biogenesis. The results could be extended to other precursors, suggesting the existence of common features in at least part of the plant precursors.

Results and Discussion

A Random Mutagenesis Method to Study MicroRNA Processing in Plants

The current model of plant microRNA (miRNA) processing implicates the nuclear localized DICER-LIKE 1 (DCL1) in both cleavage reactions required to generate miRNA/miRNA* duplexes [4–7]. DCL1 is assisted by the double-stranded RNA (dsRNA)-binding protein HYPONASTIC LEAVES1 (HYL1) and the zinc-finger protein SERRATE [6, 8–12]. Because plant precursors are structurally variable [4] and key features are difficult to predict, we turned to an unbiased approach based on a random mutagenesis to study the sequence requirements for their processing. Random mutagenesis studies have been used in a wide variety of systems (reviewed in [13, 14]); however, they are usually restricted to bacteria, yeast, or cultured cells because of the challenges underlying the screening of the mutant libraries.

We focused on miR172, which regulates AP2-like transcription factors involved in the control of flowering time and floral patterning in *Arabidopsis thaliana* [15–19]. miR172 overexpression causes early flowering [15, 16], a trait that can be easily scored by the number of rosette leaves. We first determined the minimum size of a *MIR172a* precursor that was fully active in plants. We found that the overexpression of a 145 nucleotide (nt) precursor was sufficient to cause early flowering in all of the transgenic lines (see Figure S1 available online).

We introduced random mutations along this precursor with the exception of the 21 nt miRNA sequence (see Supplemental Experimental Procedures). Therefore, the mutations could affect miR172 biogenesis but not the miRNA-target interaction. The mutated precursors were cloned under the control of a 35S viral promoter (35S:*rnd-MIR172a*; *rnd*, *rand*om mutant) and used for bulk transformation of *Arabidopsis thaliana*. Because the number of point mutations tolerated by a precursor was not known, we prepared two 35S:*rnd-MIR172a* libraries with an average of 1–2 and 4–6 mutations per precursor (Figure S2).

Control plants, transformed with an empty vector, flowered mostly with nine rosette leaves (Figure 1A). This is slightly earlier than wild-type *Arabidopsis* plants, Columbia ecotype, and is likely explained by the stress caused during the selection of the transgenics. In contrast, 35S:*MIR172a* transgenics were typically flowering with two leaves, with a minor contribution of plants with up to five leaves (Figure 1B). Importantly, the distribution of flowering time in control or 35S:*MIR172a* plants did not overlap (Figures 1A and 1B).

Primary transformants of the 35S:*rnd-MIR172a* library with an average of 1–2 mutations per precursor were mostly early flowering, though we found a subset of them with many leaves, such as those transformed with an empty vector (Figures 1C and 1D). If a 35S:*rnd-MIR172a* transgenic plant flowered with more than five leaves, we considered it to harbor a mutant precursor that was at least partially impaired in miR172 biogenesis, and it was classified as a *MIR172a* suppressor.

Sequence analysis of the 35S:*rnd-MIR172a* transgenic plants revealed that precursors without any mutation caused, as expected, early flowering (Figure 1E), whereas one mutation already suppressed *MIR172a* activity in ~15% of the cases (Figure 1E). The chances of abolishing the *MIR172a* overexpression phenotype increased with the number of mutations per precursor, and four changes were detrimental in ~75% of the cases (Figure 1E). In good agreement, most of the transgenics of the 35S:*rnd-MIR172a* library with 4–6 mutations per precursor had a suppressed *MIR172a* activity (data not shown).

Identification of Potentially Relevant Positions for *MIR172a* Processing

So far, we had selected 692 independent 35S:*rnd-MIR172a* plants. We analyzed and sequenced 220 individuals that were early flowering and 125 plants in which *MIR172a* activity was fully or partially suppressed. To assign the potential role of each precursor base, we defined rules for the interpretation of our data. Single mutations that were present in active precursors were considered neutral. If two or more mutations were introduced in a precursor causing early flowering and they corresponded to positions of the foldback that do not

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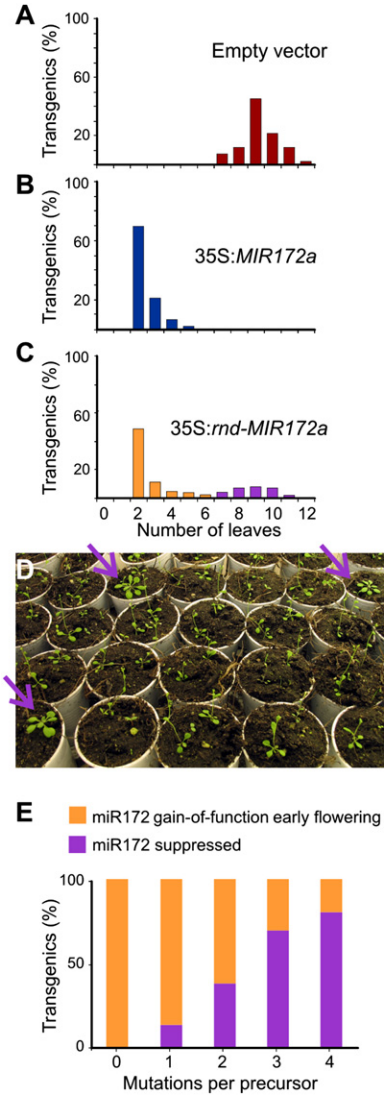

Figure 1. Generation of Transgenic Plants Expressing *MIR172a* with Random Mutations

(A–C) Distribution of flowering time in transgenic plants harboring the empty vector (A), a 35S:*MIR172a* transgene (B), and *Arabidopsis* plants bulk transformed with a random mutant library of *MIR172a* (35S:*rnd-MIR172a*), with an average of 1–2 mutations per precursor (C). Note that in the latter case, some of the transgenic plants flowered like the control (purple bars). At least 100 independent transgenic plants were analyzed in each case.

(D) A typical view of 35S:*rnd-MIR172a* primary transformants. Most of the plants are early flowering like wild-type 35S:*MIR172a*, although a few have a *MIR172a*-suppressed phenotype (indicated by arrows).

(E) Flowering time of 35S:*rnd-MIR172a* plants with a different number of mutations per precursor.

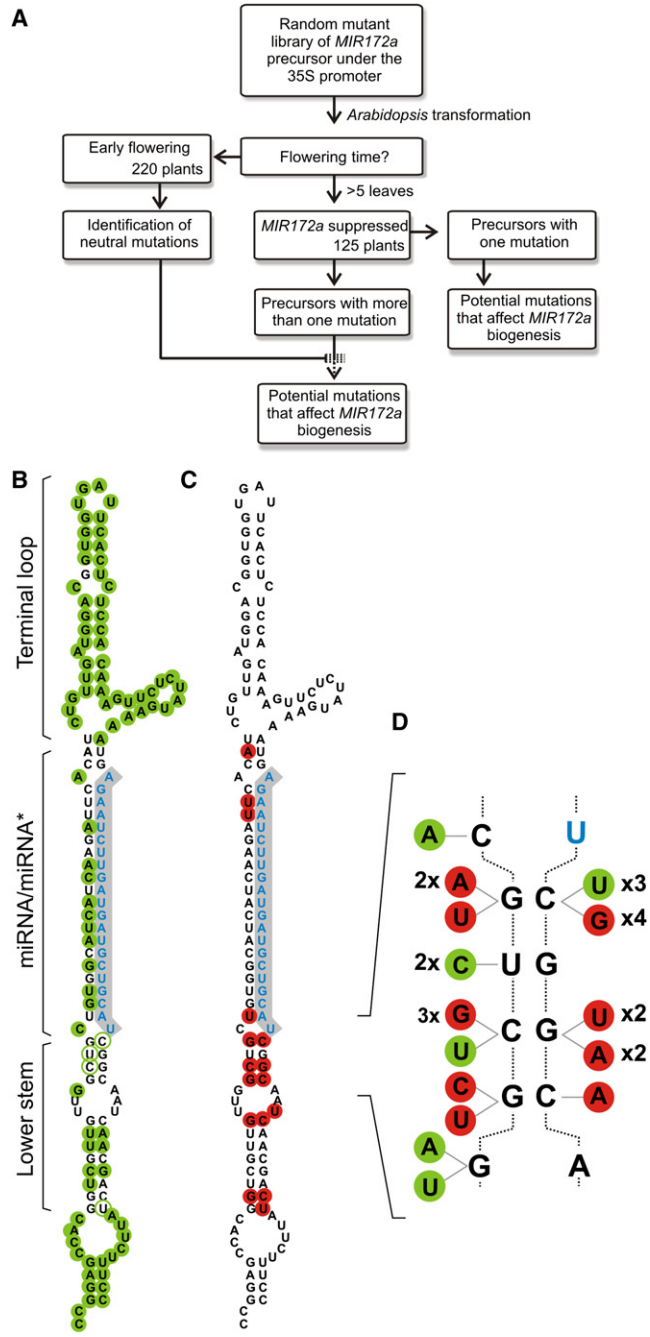

Figure 2. Analysis of Random Mutations Introduced on *MIR172a*

(A) Flow chart describing the protocol used to analyze the random mutant library. *MIR172a*-suppressed phenotype was defined as transgenic plants flowering with more than five leaves.

(B) Potential neutral bases for miR172 biogenesis are indicated with a green circle. Positions that are neutral only if the secondary structure is maintained after they are changed are indicated with open circles. Regions of the *MIR172a* precursor are indicated next to its scheme.

(C) Potential positions that are relevant for miR172 biogenesis are indicated with red circles.

(D) Close-up of a section of the lower stem of *MIR172a*. The number of mutants sequenced with each specific change is indicated.

interact between each other, we considered all of them neutral, too (Figure 2A).

Relevant positions for the precursor processing were identified from single mutations that at least partially suppressed the *MIR172a* overexpression phenotype (i.e., flowered with more than five leaves). If two or more mutations were present in one suppressed precursor, we contrasted these bases with those that were previously selected as neutral. In the cases where all except one base were already classified as neutral, the remaining base was considered relevant for miR172 biogenesis (Figure 2A). Otherwise, we were unable to assign a specific potential role to each mutation and did not continue with their study at this stage.

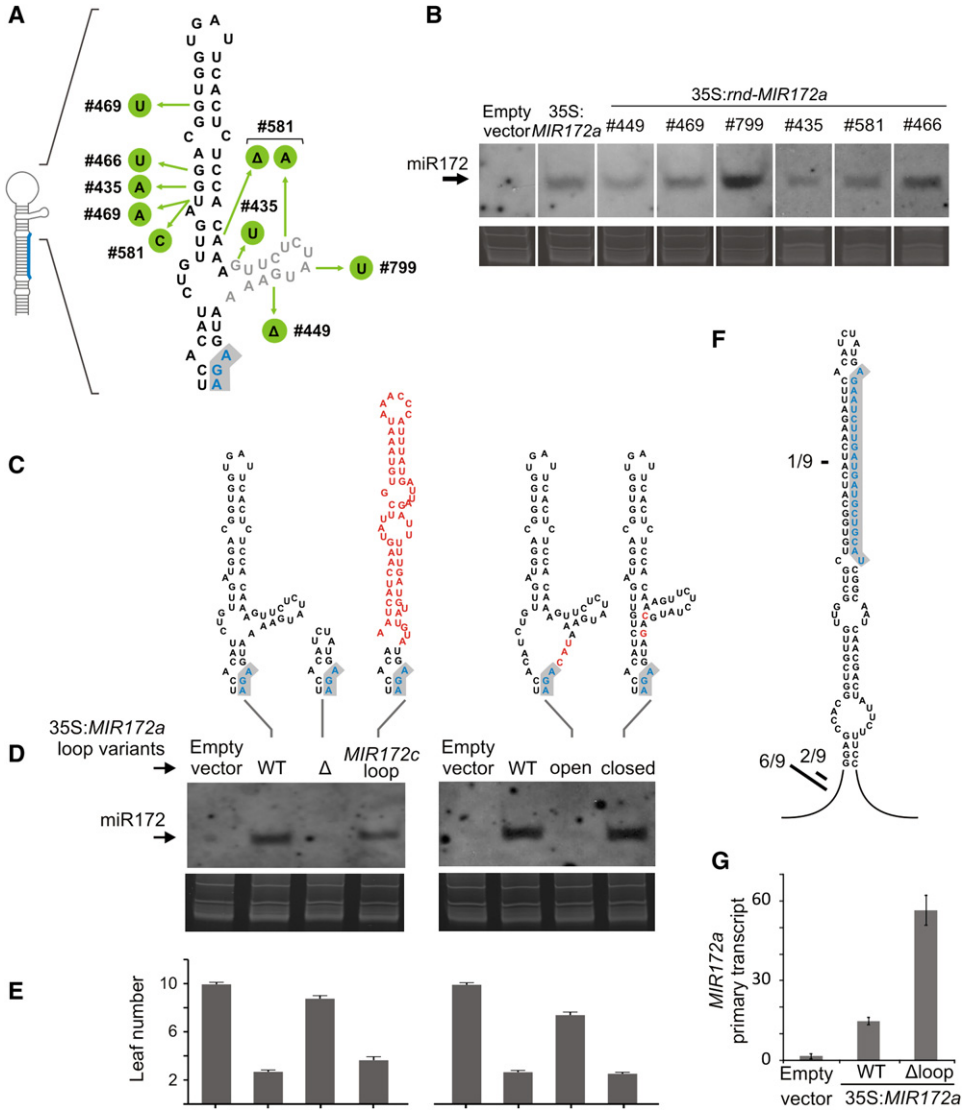

Figure 3. Role of the Loop in the Biogenesis of miR172

(A) Scheme representing a selected group of random mutants in the loop. Mutant #449 has a deletion in the bases indicated in gray.

(B) Impact of the mutations depicted in (A) on miR172 accumulation.

(C) Scheme representing several *MIR172a* loop mutants.

(D and E) Accumulation of miR172 (Δ, deleted loop precursor) (D) and flowering time (E) of the mutants obtained in (C). The data shown are mean ± standard error of the mean (SEM) of 40 plants.

(F) Cleavage intermediates for the *MIR172a* precursor with a loop deletion determined by the rapid amplification of 5′ cDNA ends (5′ RACE) polymerase chain reaction (PCR) method. The positions as revealed by 5′ RACE and the number of sequenced clones corresponding to each site are indicated by lines.

(G) *MIR172a* primary transcript levels (arbitrary units) in transgenic plants overexpressing wild-type and a deleted loop version of *MIR172a*. The data shown are mean ± SEM of three biological replicates.

With this sequence information, we constructed a map of *MIR172a* highlighting putative relevant and neutral positions for its processing (Figures 2B and 2C). As expected, mutations found to affect *MIR172a* processing localized in positions different from those considered to be neutral (Figures 2B and 2C). The precursor terminal loop contained mostly neutral positions (Figure 2B). In contrast, bases next to DCL1 cleavage sites, as well as those in the lower stem below the miRNA/miRNA* region, seemed to be most relevant for *MIR172a* processing (Figures 2C and D).

In many cases, we obtained redundant information for each position that provided robustness to our results (Figure 2D). We also validated ten random mutants by site-directed mutagenesis, confirming our previous observations (Figure S3). Results obtained in human miRNAs have also shown that one single polymorphism could impair the precursor processing [20]. The *MIR172a* map indicates that mutations in 10%–15% of the minimal precursor sequence impaired its processing and likely have a relevant role in miRNA biogenesis.

Role of the Loop in *MIR172a* Processing

Small RNA blots confirmed that point mutations in the loop do not compromise miR172 accumulation (Figures 3A and 3B). To test whether the terminal-loop region of the *MIR172a* precursor plays any role in miR172 biogenesis, we deleted the precursor loop (Figure 3C). This mutant failed to

accumulate mature miRNA (Figure 3D) or cause changes in flowering time (Figure 3E) but accumulated miR172 primary transcript (Figure 3G). Mapping of processing intermediates of this loop-deleted precursor revealed cleavage sites in the single-stranded RNA sequences outside of the foldback (Figure 3F). In contrast, the wild-type precursor had cuts at the base of the miRNA, as expected for the first DCL1 cleavage reaction that releases the stem-loop precursor from the primary transcript (Figure S4) [21]. Then we replaced the loop of *MIR172a* with that of *MIR172c*, which has a different structure (Figure 3C). The overexpression of the chimeric precursor produced high levels of miR172 and, as expected, an early flowering time (Figures 3D and 3E).

The random mutagenesis data also indicated that interactions above the miRNA were necessary for miR172 biogenesis (Figure 2C). To test the importance of the existence of a structured region close to the second cleavage site, we introduced mutations to decrease interactions in this region (open-loop *MIR172a*) and found that it did not accumulate miRNA (Figures 3C–3E). In contrast, mutations that increased the structure of the loop did not pose a challenge for miR172 biogenesis (Figures 3C–3E).

Taken together, these results indicate that most of the structural and sequence features of the loop can be modified without affecting miR172 biogenesis; however, a loop is necessary for *MIR172a* processing, and there should be a short structured segment above the miRNA sequence.

Role of the Lower Stem in *MIR172a* Processing

Most of the mutations that suppressed the 35S:*MIR172a* early flowering phenotype were located in the lower stem, up to 15 nt below the miRNA/miRNA* sequence of the foldback (Figure 2C). Analysis of these mutations illustrated that relaxing the lower-stem secondary structure could severely compromise miR172 accumulation, whereas mutations that maintained the wild-type structure (such as G-C to G-U) accumulated high miR172 levels (Figure 2; Figures 4A and 4B).

Interestingly, a mutation at position 25 (G-25) partially suppressed *MIR172a*, as we concluded from the random mutagenesis (Figures 4A and 4B) and independent primary transformants (Figures 4C–4E), which had a partial decrease of miR172 levels and flowered with 6 to7 leaves. In contrast, a change in its interacting base, namely C-122, completely suppressed miRNA accumulation (Figures 4C–4E). These differences could be explained by the concomitant rearrangements in the secondary structure of the lower stem that are prompted by the change in C-122 but not G-25 (Figure S4).

In good agreement, a deletion of the U at position 128 (U-128) also caused a large rearrangement of the lower stem and suppressed *MIR172a* activity (Figures 4C–4E). Because the lower stem is only partially structured, it is possible that a single change would allow the bases at the bulges to compete for a different folding. Therefore, the identity of the particular mutation would be important for the final precursor structure and function.

Sequence Requirements for the Biogenesis of Other miRNAs

Next, we analyzed whether other *Arabidopsis* precursors encoding conserved miRNAs have structural features similar to those found to be important for *MIR172a*. We observed that 72% of the precursors had a 15–18 nt partially structured lower stem (Table S1). To evaluate whether this region was important for the biogenesis of other miRNAs, we studied *MIR164c* and *MIR398a*, which regulate organ boundaries during plant development [22, 23] and the response to environmental signals [24, 25], respectively.

We introduced one mutation in the lower stem of *MIR164c* and two in *MIR398a* (Figures 4F and 4G). In both cases, we found a severe effect on the precursor processing (Figures 4F and 4G). We also opened the 3 nt stem above the miR398/miR398* duplex and found a deleterious impact on miR398 accumulation, albeit to a lesser extent than that caused by mutations in the lower stem (Figure 4G). In summary, our results indicate that the structural determinants deduced from the *MIR172a* studies could also be applied to other miRNAs.

Conclusions

Animal primary transcripts contain embedded uniform foldbacks that consist of an ~11 nt lower stem followed by an ~22 nt miRNA/miRNA* segment and the terminal loop [3, 26]. The Drosha-DGCR8/Pasha complex recognizes the transition between the single-stranded RNA and the stem sequences to produce the first cleavage reaction that separates the lower stem from the base of the miRNA [3]. Here, we observed that an ~15 nt lower stem, which is a common feature of many plant precursors, is a key element for miRNA biogenesis. In contrast, the loop region was largely tolerant to mutations, yet a structured segment above the miRNA sequence is required for the processing of plant precursors.

Components of the plant processing machinery, such as DCL1, HYL1, or SERRATE [4–12, 27], would likely recognize the length and degree of structure of the different precursor regions so that processing occurs in a productive way. Interestingly, the long *MIR319* and *MIR159* precursors, which lack a clear lower stem, are processed through a noncanonical loop-to-base mechanism [28], suggesting that other mechanisms might operate on precursors without *MIR172a* determinants.

Finally, by using *MIR172a* as proof of principle, we have set up a random mutagenesis approach to study sequence requirements for miRNA processing in vivo. This strategy could potentially be used for other systems in plants, especially if the sequences under study are short and a robust screening method is available.

Experimental Procedures

Plant Material

Arabidopsis thaliana Columbia ecotype was used for all experiments. Plants were grown in soil during long days (16 hr light/8 hr dark) at 23°C in a growth room. Transformation was performed via the floral dip method [29]. Transgenic plants were selected on Murashige and Skoog medium with 50 μg/ml kanamycin and transplanted to soil. Plants used for miR398 assays were grown with 10 μM $CuSO_4$ to repress endogenous miR398 [25].

Random Mutagenesis of *MIR172a* Precursor

Random mutagenesis was carried out on 124 bases out of 145 of the *MIR172a* precursor sequence. The library was constructed by primer overlap with four oligos synthesized with a nonequimolar mixture of bases [30, 31] (see Supplemental Experimental Procedures for details; see also Table S2) by using three rounds of polymerase chain reaction (PCR). These spiked oligos contained at each position the base found in the wild-type precursor at a certain frequency (w), whereas the rest of the bases were equally represented at a lower rate. Two libraries were built with different oligo sets (Figure S2) [31]. The random library was cloned under the control of *35S* promoter and used for transformation of *Arabidopsis*. Genomic DNA extracted from leaves was amplified with vector primers, and the PCR products were gel purified and sequenced. A web-based tool in Perl with MySQL (http://www.mysql.com) was

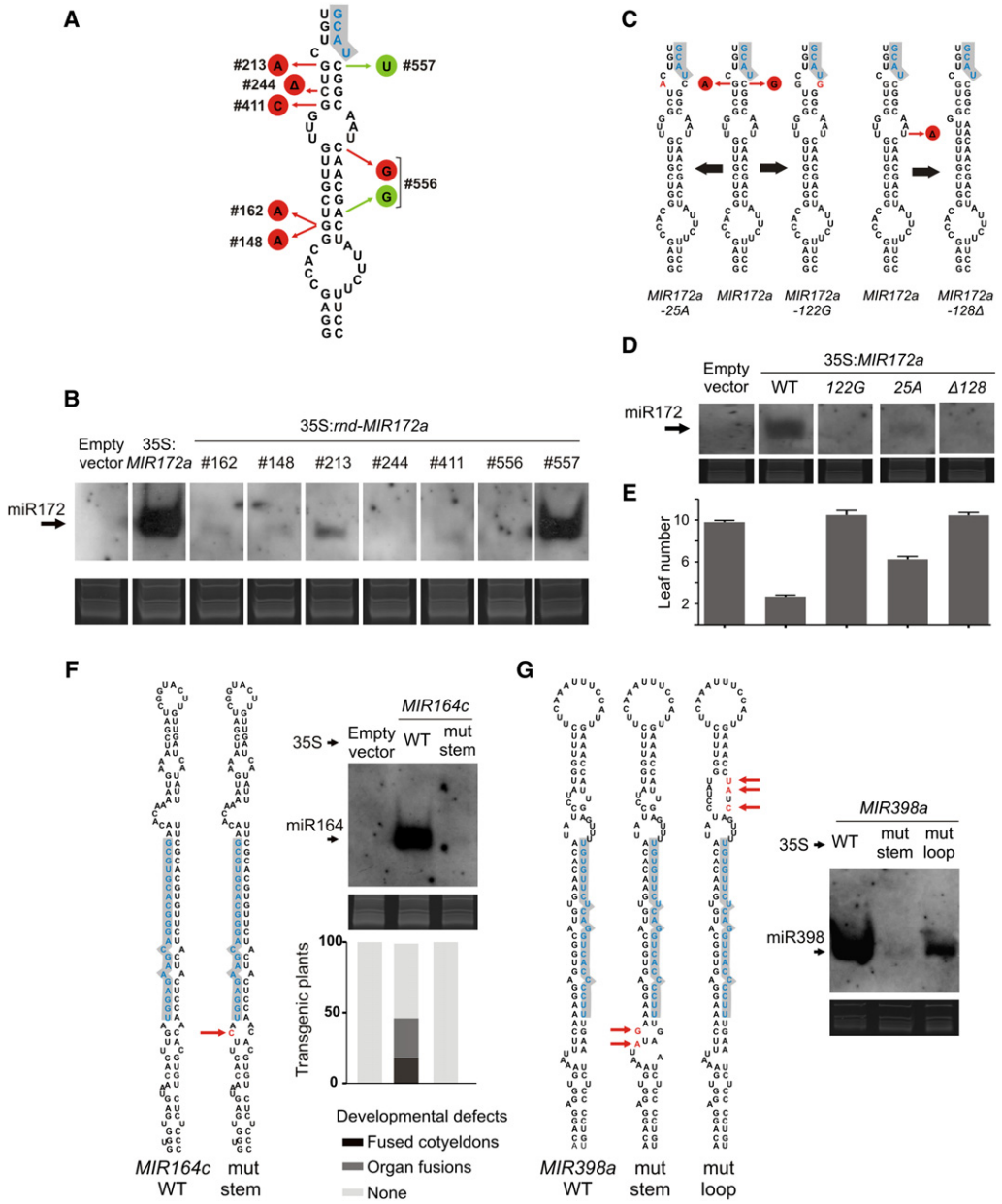

Figure 4. Role of the Lower Stem in *MIR172a* Processing

(A) Scheme of the lower stem of *MIR172a* showing a selected group of random mutants.

(B) Small RNA blots from seedlings of the mutants depicted in (A). Each sample is a pool of 25 T2 seedlings.

(C) Scheme showing the mutations between the interacting G-25 C-122 pair of the lower stem of *MIR172a* and the deletion of U-128 of the *MIR172a* precursor.

(D and E) Small RNA blots for miR172 (D) and flowering time (E) of the mutants depicted in (C). The data shown are mean ± SEM of at least 30 plants.

(F) Scheme of *MIR164c* and a mutated version (indicated in red). Right: small RNA blots of transgenic plants expressing the *MIR164* constructs. Bottom right: frequency of developmental defects in 60 transgenic lines overexpressing each construct.

(G) Scheme of *MIR398a* and mutated versions (indicated in red). Right: small RNA blots of transgenic plants expressing the different constructs.

developed to facilitate the analysis of the random mutagenesis that integrated the gathered sequence data with the precursor secondary structure and the phenotype of the transgenic plants.

Analytical Procedures

Site-directed mutagenesis on *MIR172a*, *MIR398a*, and *MIR164c* was performed by PCR (see Table S3 for a list of the constructs). The phenotypes of at least 30 independent transgenic plants were analyzed for each construct. The rapid amplification of 5′ cDNA ends (5′ RACE) method to detect cleavage fragments was carried out as described previously [32]. The primary transcript of miR172 was determined by reverse transcriptase-quantitative PCR (RT-qPCR) as described previously [33].

RNA Analysis

Total RNA was extracted from 25 seedlings corresponding to T2 transgenics (*MIR172a* random mutagenesis) or independent T1 transgenic lines (mutants obtained by site-directed mutagenesis) for each construct with TRIzol reagent (Invitrogen). Blots were hybridized with nonradioactively labeled LNA probe against miR172 and DNA probes against miR164

and miR398. The secondary structures of miRNA precursors were predicted with the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at doi:10.1016/j.cub.2009.10.072.

Acknowledgments

The seminal idea of applying a random mutagenesis to study sequence function in plants came from discussions with D. Weigel, who was interested in the analysis of the transcription factor LEAFY. We thank L. Song, N.V. Fedoroff, and J. Carrington for sharing unpublished data; V. Perdomo for technical assistance; J. Boisbouvier, R. Rasia, and D. Paladini for stimulating discussions; and C. Schommer and E. Bresso for critiques of the manuscript. J.L.M. and N.G.B. are Consejo Nacional de Investigaciones Científicas y Técnicas fellows, and J.F.P. is a member of the same institution. This work was supported by grants from the Human Frontier Science Program Organization Young Investigators Program and the international program from Howard Hughes Medical Institute to J.F.P.

Received: August 24, 2009
Revised: October 14, 2009
Accepted: October 29, 2009
Published online: December 10, 2009

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